Two-photon laser scanning microscopy

Publication number: EP0807814
Publication date: 1997-11-19
Inventor: DENK WINFRIED (CH); STRICKLER JAMES P (US); WEBB WATT W (US)
Applicant: CORNELL RES FOUNDATION INC (US)
Classification:
- international: ***G01N21/64; G02B21/00; G11B7/004; G11B7/12; G11B7/135;*** **G01N21/64; G02B21/00; G11B7/00; G11B7/12; G11B7/135;** (IPC1-7): G01N21/64; G02B21/00; G11B7/00
- european: G01N21/64H; G02B21/00M4A7U; G11B7/004; G11B7/12; G11B7/135D
Application number: EP19970108581 19901113
Priority number(s): EP19900917225 19901113; US19890436045 19891114

Also published as:
EP0807814 (B1)

Cited documents:
US4288861
GB1236108
XP002035947
XP002035948
XP002035949
more >>

Abstract of **EP0807814**

A method and apparatus for molecular excitation causes such excitation by submicrosecond, rapidly repeating pulses of coherent photons of an energy less than that needed for the excitation, which are focussed so that two-photon combination occurs at a determined site. Accurate control of chemical reaction in biological specimens, or actuation of optical memories, become possible.

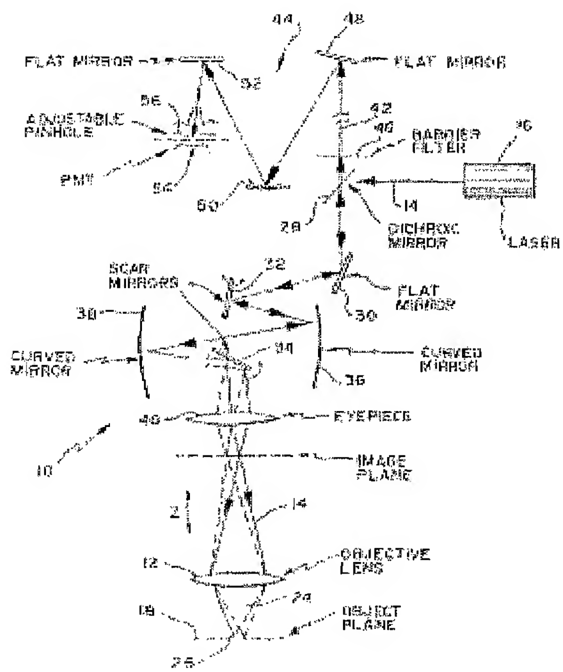


FIG. 1

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 807 814 A1

(12) # EUROPEAN PATENT APPLICATION

(43) Date of publication:
19.11.1997 Bulletin 1997/47

(51) Int Cl.6: **G01N 21/64**, G02B 21/00, G11B 7/00

(21) Application number: **97108581.6**

(22) Date of filing: **13.11.1990**

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB GR IT LI LU NL SE

(30) Priority: **14.11.1989 US 436045**

(62) Document number(s) of the earlier application(s) in accordance with Art. 76 EPC:
90917225.6 / 0 500 717

(71) Applicant: **CORNELL RESEARCH FOUNDATION, INC.**
Ithaca, NY 14850 (US)

(72) Inventors:
- **Denk,Winfried, c/o IBM Forschungslaboratorium 8803 Ruschlikon (CH)**
- **Strickler,James P. Ithaca, New York 14850 (US)**
- **Webb,Watt W. Ithaca, New York 14850 (US)**

(74) Representative: **Harrison, David Christopher et al**
MEWBURN ELLIS
York House
23 Kingsway
London WC2B 6HP (GB)

Remarks:
This application was filed on 28 - 05 - 1997 as a divisional application to the application mentioned under INID code 62.

(54) **Two-photon laser scanning microscopy**

(57) A method and apparatus for molecular excitation causes such excitation by submicrosecond, rapidly repeating pulses of coherent photons of an energy less than that needed for the excitation, which are focussed so that two-photon combination occurs at a determined site. Accurate control of chemical reaction in biological specimens, or actuation of optical memories, become possible.

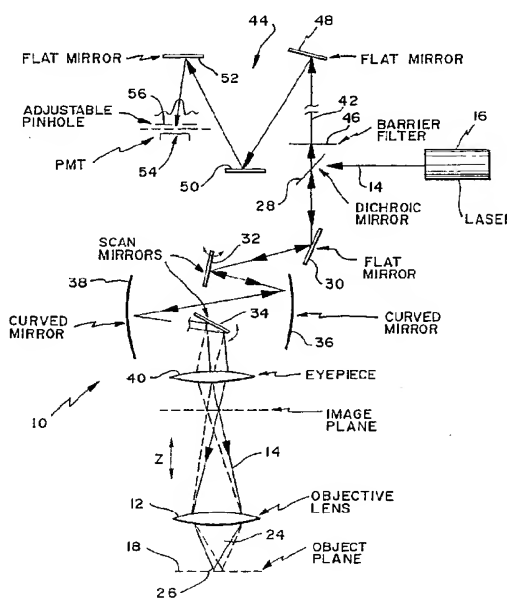


FIG. 1

EP 0 807 814 A1

Description

BACKGROUND OF THE INVENTION

This invention was made with Government support under Grant Nos. P41RR04224 awarded by the National Institutesof Health; NSF-BBS-8714069 awarded by the National Science Foundation, and NSF-DMB-8609084 awarded by the National Science Foundation. The United States Government has certain rights in the invention.

Although the principle of a flying spot scanner has been known for many years, its application in microscopy has prospered only in the last few years as the necessary technology has been developed. Stable laser light sources and fast electronic image acquisition and storage technology are necessary ingredients for a scanning microscope. While the imaging properties of a non-confocal scanning microscope are very similar to those of conventional microscopes, a new domain is opened by confocal scanning microscopes. The resolution provided by such devices is only moderately increased, but the vastly improved depth discrimination they provide allows the generation of three dimensional images without complicated deconyolution algorithms. The depth discrimination reduces background, and this, together with the use of a single high quality detector such as a photomultiplier, allows quantitative studies with high spatial resolution.

The resolution along the optical axis of a confocal scanning microscope provides useful discrimination against background scattering or fluorescence arising above and below the plane of focus in a transparent object. It is also very helpful in constructing three dimensional fluorescent images from a series of sections and for the use of quantitative fluorescence indicators or for mapping of fluorescent markers of cell surface receptors on nonplanar surfaces. Such devices provide slightly better lateral resolution, much better depth field discrimination, and orders of magnitude better background discrimination under ideal conditions than was available with prior devices, under ideal conditions.

Scanning can be carried out either by moving the specimen stage under a stationary beam or by precisely synchronized optical scanning of both the illumination and the fluorescent response signals. Although the moving stage solution is preferable from an optical point of view, it puts limits on sample access and mounting, the use of environmental chambers, and electrical recording with microelectrodes. Accordingly, the moving spot approach is often favored. Such a moving spot may be produced by the use of mirrors mounted on galvanometer scanners, although this limits the obtainable frame frequency. The use of accousto-optical deflectors interferes with the confocal spatial filtering in fluorescence microscopy because of their strong dispersion. Although polygonal mirrors are faster than galvonometer scanners, one alone does not allow a vector mode of operation.

A conventional arc light source can be used for many applications of a confocal scanning microscope which utilizes a rotating disc illuminator, but apparently inescapable intensity modulations limit its use for quantitative applications. In such devices, the image is formed either through a dual set of confocal pin holes in the disc, or, in recent versions, through the illumination pinholes themselves.

Confocal scanning microscopes in which a single point illuminated by a laser is scanned across the moving object work quite well at slow scanning speeds, and good laser scanning micrographs have been obtained using fluorescence markers that absorb and emit visible light. However, confocal scanning images with fluorophores and fluorescent chemical indicators that are excited by the ultraviolet part of the spectrum have not been available, largely because of the lack of suitable microscope lenses, which must be chromatically corrected and transparent for both absorption and emission wavelengths, but also because of the damage done to living cells by ultraviolet light. Furthermore, the limitations of ultraviolet lasers have inhibited such usage. Fluorescence microscopy is further limited, in all of its manifestations, by the photobleaching of fluorophores in the target material, for the exciting light slowly photobleaches the fluorophores while it is exciting fluorescence. Even in laser scanning confocal fluorescence microscopy, essentially the same photobleaching is incurred as happens in wide field microscopy, because the focused exciting light still illuminates the full depth of the target specimen uniformly, in a time average, as it scans the plane of focus. Photobleaching is particularly troublesome in a three-dimensional image reconstruction because many two-dimensional images are required for this purpose, and the acquisition of each two-dimensional image produces photobleaching throughout the specimen.

SUMMARY OF THE INVENTION

The foregoing difficulties are overcome, in accordance with the present invention, by the use of two-photon molecular excitation of fluorescence in laser scanning microscopy. Two-photon excitation is made possible, in accordance with the present invention, by the combination of (a) the very high, local, instantaneous intensity provided by the tight focusing available in a laser scanning microscope, wherein the laser can be focused to a diffraction-limited waist of less than 1 micron in diameter, and (b) the temporal concentration of a pulsed laser. A high intensity, long wavelength, monochromatic light source which is focusable to the diffraction limit such as a colliding-pulse, mode-locked dye laser, produces a stream of pulses, with each pulse having a duration of about 100 femtoseconds (100×10^{-15} seconds) at a repetition rate of about 80 MHz. These subpicosecond pulses are supplied to the microscope, for example by way of

a dichroic mirror, and are directed through the microscope optics to a specimen, or target material, located at the object plane of the microscope. Because of the high instantaneous power provided by the very short duration intense pulses focused to the diffraction limit, there is an appreciable probability that a fluorophore (a fluorescent dye), contained in the target material, and normally excitable by a single high energy photon having a short wavelength, typically ultraviolet, will absorb two long wavelength photons from the laser source simultaneously. This absorption combines the energy of the two photons in the fluorophore molecule, thereby raising the fluorophore to its excited state. When the fluorophore returns to its normal state, it emits light, and this light then passes back through the microscope optics to a suitable detector.

The two-photon excitation of fluorophores by highly intense, short pulses of light constitutes a general fluorescence technique for microscopy which provides improved background discrimination, reduces photobleaching of the fluorophores, and minimizes the photo damage to living cell specimens. This is because the focused illumination produced in the microscope fills a converging cone as it passes into the specimen. All of the light which reaches the plane of focus at the apex of the converging cone, except the tiny fraction which is absorbed in the fluorophore, then passes out the opposite side of the specimen through a diverging cone. Only in the region of the focal point on the object plane at the waist formed by the converging and diverging cones is the intensity sufficiently high to produce two photon absorption in the specimen fluorophore, and this intensity dependence enables long wavelength light to provide the effect of short wavelength excitation only in the small local volume of the specimen surrounding the focal point. This absorption is produced by means of a stream of fast, high intensity, femtosecond pulses of relatively long wavelength which retains a moderate average illumination intensity of long wavelength light throughout the remainder of the specimen outside the region of the focal point. As a result, photobleaching of the fluorophore outside the plane of focus is virtually eliminated. One-photon absorption of the long wavelength light is negligible, and outside the plane of focus the instantaneous intensity is too low for appreciable two-photon absorption and excitation, even though the time average illumination is in reality nearly uniform throughout the depth of the specimen. This effect also significantly reduces the damage to living cells.

The two-photon excitation of the present invention allows accurate spatial discrimination and permits quantitation of fluorescence from small volumes whose locations are defined in three dimensions, and thus provides a depth of field resolution comparable to that produced in confocal laser scanning microscopes without the disadvantages of confocal microscopes previously described. This is especially important in cases where thicker layers of cells are to be studied. Furthermore, the two-photon excitation greatly reduces the background fluorescence.

The two-photon absorption technique discussed above can also be used to excite selected locations in a three-dimensional optical memory device of the type described by Dimitri A. Parthenopoulos et al in an article entitled "Three-dimensional Optical Storage Memory", Science, Vol. 245, pages 843-845, August 25, 1989. In accordance with the present invention, extremely short, high intensity pulses of relatively long wavelength light from a single laser source, or from coaxial multiple sources, are directed through a scanning microscope into a storage medium which may be a photochromic or a photolyzable fluorescent material such as crystals, composites, or chomophores embedded in a polymer matrix. The incident light beam is highly focused onto any one of many layers in the matrix, and its intensity is modulated as it is scanned or stepped across the selected layer. The beam excites selected locations in the matrix so that coded information represented by the beam is stored in a binary format within the medium. The highly focused beam provides the spatial resolution required for accurate storage. The femtosecond, high intensity pulses induce two-photon absorption in the matrix material to write information into the material, which normally requires excitation by light in the ultraviolet range. The excitation level of the written points in the matrix can be detected, or read, by a "read" laser of long wavelength which will produce fluorescence in the previously written molecules.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing, and additional objects, features and advantages of the present invention will become apparent from the following detailed description of preferred embodiments thereof, taken in conjunction with the accompanying drawings, in which:

Fig. 1 is a diagrammatic illustration of a laser scanning confocal microscope utilized in accordance with the present invention;
Fig. 1A is an enlarged partial view of the region of the object plane of the device of Fig. 1;
Fig. 2 is a synthesized stereo image pair showing blue fluorescence excited by two-photon absorption of red light;
Fig. 3 is a plot of the average intensity from an area inside a fluorescent latex bead versus the applied average laser power;
Fig. 4 is a two-photon excited fluorescence image of chromosomes of live cultured pig kidney cells stained with a DNA stain;
Fig. 5 is an image of a latex bead, showing two-photon photobleaching confined to the plane of focus; and

Fig. 6 is an image of a two-photon bleached pattern inside a fluorescently stained latex bead.

DESCRIPTION OF PREFERRED EMBODIMENT

Turning now to a more detailed description of the present invention, there is illustrated in Fig. 1 in diagrammatic form a conventional laser scanning microscope 10 which includes an objective lens 12 for focusing incident light 14 from a source 16 such as a laser onto an object plane 18. As illustrated in Fig. 1A, the object plane may lie on, or in, a specimen or target material 20 which may be carried on a movable stage 22. The illumination provided by incident light beam 14 fills a converging cone generally indicated at 24, the cone passing into the specimen 20 to reach the plane of focus at object plane 18 and, except for the tiny fraction of light absorbed by the specimen, passing out through a diverging cone 25. The incident light forms a waist, or focal point, 26 on the object plane 18. The diameter of the focal point 26 is limited by diffraction in the optical path, but preferably is less than 1 micron. As is known, by adjustment of the microscope optics, the vertical location of the focal point in the specimen 20 can be selected. Additionally, the stage 22 may be movable in a horizontal plane, as in a raster motion along X and Y axes, to position the incident light at selected locations in the specimen in the horizontal plane, so that three-dimensional scanning of the specimen can be obtained. However, since mechanically scanned stages present difficulties, it is preferred to use a stationary stage, and to scan the incident beam in the X-Y plane optically, as by means of scanning mirrors in the optical path of the microscope.

The optical path from laser 16 to the object plane 18 includes a dichroic mirror 28 onto which the light from the laser 16 is directed. As will be explained in greater detail below, in accordance with the present invention the output from the laser consists of short intense pulses of light having a relatively long wavelength, preferably in the visible red or near infrared spectral range. The mirror 28 deflects this long wavelength light downwardly to a mirror 30 which in turn directs the light to a pair of scanning mirrors 32 and 34 by way of curved mirrors 36 and 38. The mirrors 32 and 34 are rotatable about mutually perpendicular axes in order to move the incident light 14 along perpendicular X and Y axes on the object plane so that the stationary specimen is scanned by the incident beam. The light from the scanning mirrors passes through eyepiece 40 and is focused through the objective lens 12 to the object plane 18.

Fluorescence produced in the specimen 20, indicated by dotted arrows 42 in Fig. 1A, travels back through the microscope 10, retracing the optical path of the incident beam 14, and thus passes through objective lens 12 and eyepiece 40, the scanning mirrors 34 and 32 and the curved mirrors 38 and 36, and is reflected by mirror 30 back to the dichroic mirror 28. The light emitted by fluorescent material in the specimen is at a wavelength that is specific to the fluorophore contained in the specimen, and thus is a different wavelength than the incident light 14. This fluorescent light is able to pass through the dichroic mirror 28, rather than being reflected back toward the laser 16, and follows the light path indicated generally at 44. The fluorescent light 42 thus passes through a barrier filter 46 and is reflected by flat mirrors 48, 50 and 52 to a suitable detector such as a photomultiplier tube 54. In accordance with the present invention, a confocal laser scanning microscope is preferred, and accordingly such a microscope is illustrated in the drawings. However, it will be understood that other laser scanning microscopes may be used. In the confocal microscope 10, an adjustable confocal pin hole 56 is provided in the collection optics 44 to minimize background fluorescence excited in the converging and diverging cones 24 and 25 above and below the plane of focus. This confocal pinhole is useful, but is not necessary in the two photon fluorescence excitation of the present invention, since excitation is essentially limited to the region of the focal point 26 on the object plane.

With prior fluorescence microscopes the visible light fluorescence photons 42 are produced by molecules that are excited by absorbing a single photon from incident light 14 that has higher energy; that is, a shorter wavelength, than the fluorescence 42 generated during relaxation of the molecule from its excited state. The number of fluorescence photons released per molecule in such prior devices is ordinarily linearly proportional to the number of exciting photons absorbed. Because only a single photon need be absorbed in such devices, photolysis of molecules that absorb the exciting light 14 can occur all along the double cone beam 24 and 25 within the specimen 20, although this process is not necessarily linear with intensity. Because fluorescence is generated all along the double cone beam, the amount of fluorescence released from each plane in the specimen above, below and within the plane of focus of the exciting light 14 tends to be the same, and three dimensional resolution is difficult to obtain. As a result, the high energy of the incident light throughout the specimen tends to damage the specimens and this is particularly undesirable when living cells are being viewed.

In order to obtain three dimensional resolution in scanning microscopy and to reduce damage to the specimen in regions outside the focal point of the microscope, the present invention utilizes two-photon excitation of a fluorophore which has a one-photon absorption peak at a wavelength which overlaps one-half that of the exciting light. To accomplish this, the laser 16 produces a very short pulsed laser beam of high instantaneous power and of a relatively long wavelength, for example in the visible red or the infrared range. This light is directed to a specimen containing a fluorophore normally excited by a single photon in the short wavelength, for example ultraviolet, range so that two low energy (red) photons must combine their energy to provide the same excitation of the specimen that would be provided

by a single high energy (ultraviolet) photon. Both the excitation and hence the fluorescence rates in the specimen are proportional to the square of the intensity of the incident light. In the focused excitation laser beam 14, the intensity of the long wavelength incident light becomes high enough to excite the fluorophores in the specimen only in the region of the focal point 26 of the microscope optics. This focal point may be adjustably positioned within the specimen, so that fluorescence and/or photolysis of the specimen are produced only in a selected ellipsoidal volume around the focus. Thus, in accordance with the invention, only long wavelength excitation light has to pass through the specimen, and this long wavelength light is focused to produce sufficient intensity to excite fluorescence only in a very small region. This fluorescence is produced even if the fluorophore normally absorbs only in the ultraviolet. Since the focal point can be selectively positioned in the specimen, three-dimensional resolution is provided in both scanning fluorescence microscopy and in photolysis, including photolysis of photon-activatable reagents which can be released by photolysis.

In accordance with the present invention, the necessary excitation intensity is provided at the focal point of the microscope 10 from a light source 16 which may be, for example, a colliding pulse, mode-locked dye laser generating pulses of light having a wavelength in the red region of the spectrum, for example about 630 nm, with the pulses having less than 100 fsec. duration at about 80 MHz repetition rate. Other bright pulsed lasers may also be used to produce light at different relatively long wavelengths in the infrared or visible red region of the spectrum, for example, to generate the necessary excitation photon energies which will add up to the appropriate absorption energy band required by the fluorophores in the specimen which normally would be excited by absorption of a single photon in the spectral region having wavelengths about one-half the wavelength of the incident light. Thus, for example, two photons in the visible red region at 630 nm would combine to excite a fluorophore which normally absorbs light in the ultraviolet region at 315 nm, while two photons in the infrared region of, for example, 1070 nm, would excite a fluorophore which absorbs at 535 nm in the visible light region.

In a modified form of the invention, the single wavelength light source 16 can be replaced by two different long wavelength laser sources so that the incident light beam 14 consists of two superimposed pulsed light beams of high instantaneous power and of different wavelengths. The wavelengths of the incident beam are selected to excite a fluorophore which is absorbent at a short wavelength which may be described as:

$$^1/_{\lambda_{abs}} = {}^1/_{\lambda_1} + {}^1/_{\lambda_2}$$

where λ_{abs} is the short wavelength of the absorber, and λ_1, λ_2 are the laser incident beam wavelengths..

In two-photon excitation, with a typical two-photon cross section δ of:

$$\delta = 10^{-58}\ m^4 s/photon \qquad \text{(Eq. 1)}$$

and with the pulse parameters given above (100 fsec. pulses at a repetition rate of 80 MHz), and with the beam focused by a lens of numerical aperture A = 1.4, the average incident laser power (p_0) of approximately 50 mW saturates the fluorescence output of a fluorophore at the limit of one absorbed photon per pulse per fluorophore. The number n_a of photons absorbed per fluorophore per pulse depends on the following relationship:

$$n_a \approx \frac{P_o^2 \delta}{\tau f^2} \left[\frac{A^2}{2 \hbar c \lambda} \right]^2 \qquad \text{(Eq. 2)}$$

where

- τ is the pulse duration;
- f is the repetition rate;
- P_0 is the average incident laser power;
- δ is the photon absorption cross section;
- $\hbar$ is the Planck quantum of action;
- c is the speed of light; and
- A is the numerical aperture of the focusing lens. The fluorescence emission could be increased, however, by increasing the pulse repetition frequency up to the inverse fluorescence lifetime, which typically is:

$$\tau_f^{-1} = 10^9\ S^{-1} \qquad \text{(Eq. 3)}$$

For comparison, one-photon fluorescence saturation occurs at incident powers of about 3 mW.

Fig. 2 illustrates the depth discrimination achieved by the two photon technique of the present invention. A stereo pair of images 60 and 62 was generated from a stack of images of a cluster of fluorescent 9 micrometer diameter latex beads which are normally excited by ultraviolet light having a wavelength of about 365 nm. These images were obtained using a standard laser scanning microscope, but with its continuous-wave argon-ion laser illuminator 16 replaced by a 25 mw colliding-pulse mode-locked dyelaser producing output pulses at a wavelength of about 630 nm. Measurements made on the microscope 10 indicated that about 3 mw reached the object plane. An emission filter, passing wavelengths from 380 to 445 nm, was provided at the barrier filter 46, and the detector aperture 54 was opened to its limit in order to reduce the optical sectioning effect that would result from a small confocal aperture.

The intensity of the incident beam 14 from laser 16 was adjusted by placing neutral density filters in the excitation beam between laser 16 and the dichroic mirror 28 and the blue fluorescence produced by the individual latex beads was measured. As illustrated in Fig. 3 by the graph 64, the detected intensity of fluorescence from the latex beads making up the specimen increased with the square of the excitation laser power, clearly indicating two-photon excitation in the beads. The excitation cross section of the beads, which were "fluoresbrite BB" beads produced by Polysciences Corporation, was estimated to be 5×10^{-58} M^4 s/photon, accurate within a factor of 3, by taking into account the dye concentration in the beads, the optical throughput of the laser scanning microscope, the pulse duration, the repetition rate, the numerical aperture and the incident power. This value was found to be comparable to previously measured values for similar dyes.

Fig. 4 is a scanned image of chromosomes in dividing cells (LLC-PK1; ATTC), using cellular DNA labeling with an ultraviolet excitable fluorescent stain (33258; Hoechst) the image acquisition time of 13 seconds was short compared to the bleaching time of several minutes. Furthermore, no degradation was apparent in these live cells even after illumination by the scanning laser for several minutes.

Photobleaching during protracted scanning of a fluorescent bead occurred only in a slice about 2 micrometers thick around the focal plane, as demonstrated by the horizontal section 70 of reduced brightness bleached out of the bead 72 illustrated in Fig. 5. This bead was scanned for six minutes at a constant focal plane position. Similar localization of bleaching was observed in the fluorescently stained cell nuclei. This localization illustrates a distinct advantage over the use of single-photon excitation, where the entire specimen is bleached even when only a single plane is imaged. This is because for one-photon excitation, bleaching in both scanning and broad field microscopy depends on the time averaged excitation intensity, which does not vary along the axial, or Z-direction indicated in Fig. 1. For two-photon excitation, on the other hand, bleaching depends on the time averaged square of the intensity, which falls off strongly above and below the focal plane.

The dependence of the fluorescent signal on the square of the excitation intensity is responsible for another advantage of two-photon excitation; that is, such excitation provides an optical sectioning effect through the specimen, even when using a detector, such as a CCD array, which views the whole field, without a pinhole being used as a spatial filter. This sectioning effect, which is illustrated in Fig. 5, avoids the serious problems associated with chromatic aberration in the objective lens and some of the throughput losses in conventional confocal laser scanning microscopes.

Two-photon photolysis can also be used for fast and localized release of biologically active chemicals such as caged Ca++, H+, nucleotides and neurotransmitters. For example, when caged neurotransmitters are released by a scanning beam, the whole-cell transmembrane current so produced is usable as the contrast-generating mechanism to map the distribution of receptor activity for those transmitters on the cell surface. The feasibility of two-photon cage photolysis was demonstrated, in accordance with the present invention, by irradiating DMNPE caged ATP (33mM) [from Molecular Probes, Eugene Oregon], by the colliding pulse mode locked dyelaser 16 focused to a beam waist diameter at the object plane of about 10 micrometers. Photolysis yields of about 10^{-11} moles of ATP were measured using a luciferin bioluminescence assay from Calbiochem, San Diego, CA. Typically, about 10% of the caged ATP in an aliquot volume of about $10^7 (\mu m)^3$ was photolyzed in the illumination volume of about $10^4 (\mu m)^3$ during about 600 seconds.

Since two-photon excitation in accordance with the present invention provides access by visible light to excitation energies corresponding to single-ultraviolet-photon excitation, a whole new class of fluorophores and fluorescent indicators becomes accessible to three-dimensionally resolved laser scanning microscopy. Such indicators may be Indo-1 for Ca^{+2}, Mag-Indo-1 for Mg^{+2}, ABF1 for Na^+ and PBFI for K^+. Although two-photon cross sections are not yet known for many of these compounds, and different selection rules apply to two-photon absorption, molecular asymmetry often allows both one photon and two-photon transitions into the same excited state. Visible fluorescence was observed from 10mM solutions of Indo-1, FURA-2, Hoechst 33258, Hoechst 33342, DANSYL hydrazine [Molecular Probes], Stilbene 420 [Exciton Chem. Co., Dayton, OH], and several Coumarin dyes upon excitation by a CMP weakly focused

to a 25 m diameter waist, and two-photon excited LSM fluorescence images of microcrystals of DANSYL and Coumarin 440 were recorded.

Another application of the present invention may be in three-dimensional optical memory devices which rely on multi-photon processes in two intersecting beams for writing and reading operations. A single beam would be simpler than the two intersecting beams, and would permit maximal information packing density. The multi-photon processes would be localized to the high intensity region at the focus, as illustrated in Fig. 5 where the bleaching of microscopic patterns inside fluorescent beads constitutes a high density write once memory which is readable about 10^3 times with present fluorophores.

Thus there has been described and illustrated a practical two-photon laser scanning fluorescence microscope for biological and other applications. The two-photon excited fluorescence microscope provides inherent three-dimensional resolution with a depth of field comparable to that produced by confocal laser scanning microscopes. The use of a confocal pinhole in conjunction with this two-photon excitation further improves resolution along all three axes. Background fluorescence can be eliminated by scaled subtraction of images which are recorded at different input powers. With the present technique, photobleaching, as well as photodynamic damage, can be confined to the vicinity of the focal plane, thereby providing a considerable advantage over both confocal laser scanning microscopy and area detector imaging for the acquisition of data for three dimensional reconstruction, since ultraviolet damage to cells and fluorophores would be confined to the volume from which information is actually collected. This also allows sharp localization of photochemical processes such as photolysis and photoactivation within the focal volume. The invention is principally described as utilizing two photons from a single laser, but it should be understood that excitation of the target material can also be accomplished by two photons from two sources, as long as the two different wavelengths add up to the excitation wavelength of the target material. Thus, for example, two different laser sources could be used, with their output beams being directed coaxially into the optical path of the microscope. Alternatively, two different wavelengths could be derived from a single source, as by means of a frequency doubler.

Although the present invention has been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations and modifications may be made without departing from the true spirit and scope thereof as set forth in the accompanying claims.

Claims

1. Apparatus (10) for inducing molecular excitation in a target material (20) responsive to single photon excitation by light of a characteristic energy, said apparatus comprising

   an object plane (18) for receiving said target material (20);
   focussing means (40,12) positioned adjacent said object plane;
   a repetitive source (16) of subpicosecond light pulses, said light pulses of photons having an energy less than said characteristic energy;
   mirror means (36,38) directing said light pulses along an optical path including said focussing means (40,12) to cause said pulses to impinge on said target material (20), said focussing means (40,12) focussing said light pulses on a focal volume (26);
   so that incident photons in combination induce the excitation in the target material.

2. The apparatus of claim 1, wherein said focussing means (40,12) is adjustable to select focal volumes (26) at different depths within said target material (20).

3. The apparatus of claim 2, further including scanning means (32,34) to move said focal volume (26) with respect to said target material (20).

4. The apparatus of claim 1, claim 2 or claim 3, wherein said target material includes a biological cell responsive to said excitation.

5. The apparatus of claim 4, wherein said laser source and the optical path are adapted to scan a target material responsive to said excitation at said focal point to produce localized release of biologically active chemicals.

6. The apparatus of claim 1, claim 2 or claim 3 wherein said target material has a photon-activatable reagent.

7. The apparatus of claim 1, claim 2 or claim 3 wherein said target material has an optical memory.

8. A method of causing molecular excitation technique of a target material (20) containing molecules which are excitable by photons of a characteristic energy; by the steps of

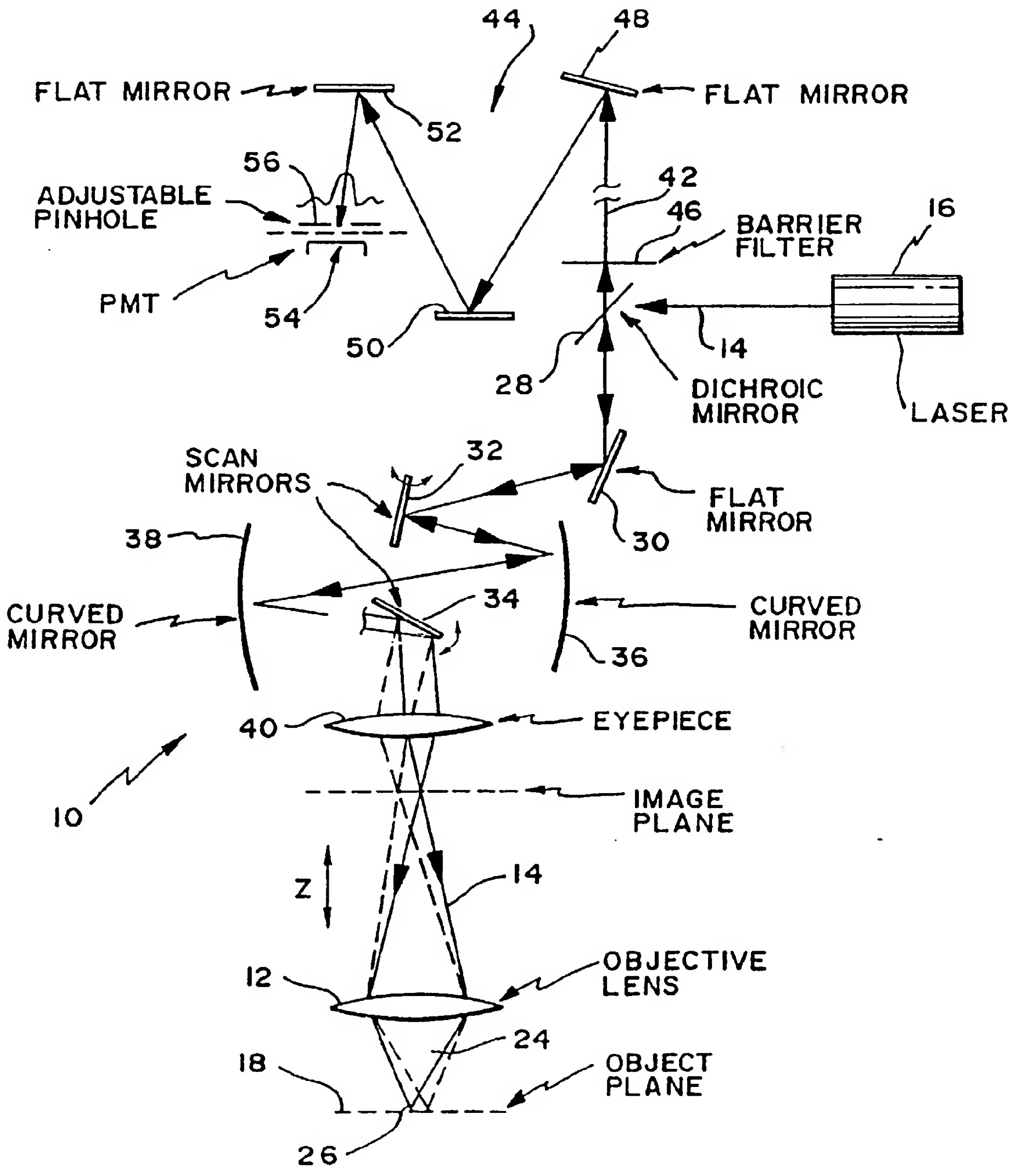
illuminating said material (20) with a beam of intense, subpicosecond pulses of laser light comprising photons of an energy less than said characteristic energy; and
focussing said illumination to a small focal volume (26) within said material (20) to produce an illumination intensity sufficiently high only at said focal volume to produce molecular excitation by simultaneous absorption of at least two of said incident illuminating photons.

9. The method according to claim 8, wherein the incident photons are of half the characteristic energy.

10. The method of claim 8 or claim 9, wherein the material (20) includes caged biologically active molecules, said illumination intensity within said focal volume (26) being sufficient to release caged biologically active compounds by simultaneous absorption of incident photons.

11. The method of claim 8 or claim 9, wherein the target material includes a biological cell responsive to the excitation.

12. The method of claim 8 or claim 9, wherein the target material is an optical memory.



44
48
FLAT MIRROR
FLAT MIRROR
52
56
42
ADJUSTABLE PINHOLE
46
BARRIER FILTER
16
PMT
54
50
14
28
DICHROIC MIRROR
LASER
SCAN MIRRORS
32
FLAT MIRROR
30
38
CURVED MIRROR
34
CURVED MIRROR
36
40
EYEPIECE
IMAGE PLANE
10
Z
14
12
OBJECTIVE LENS
24
18
OBJECT PLANE
26

FIG. 1

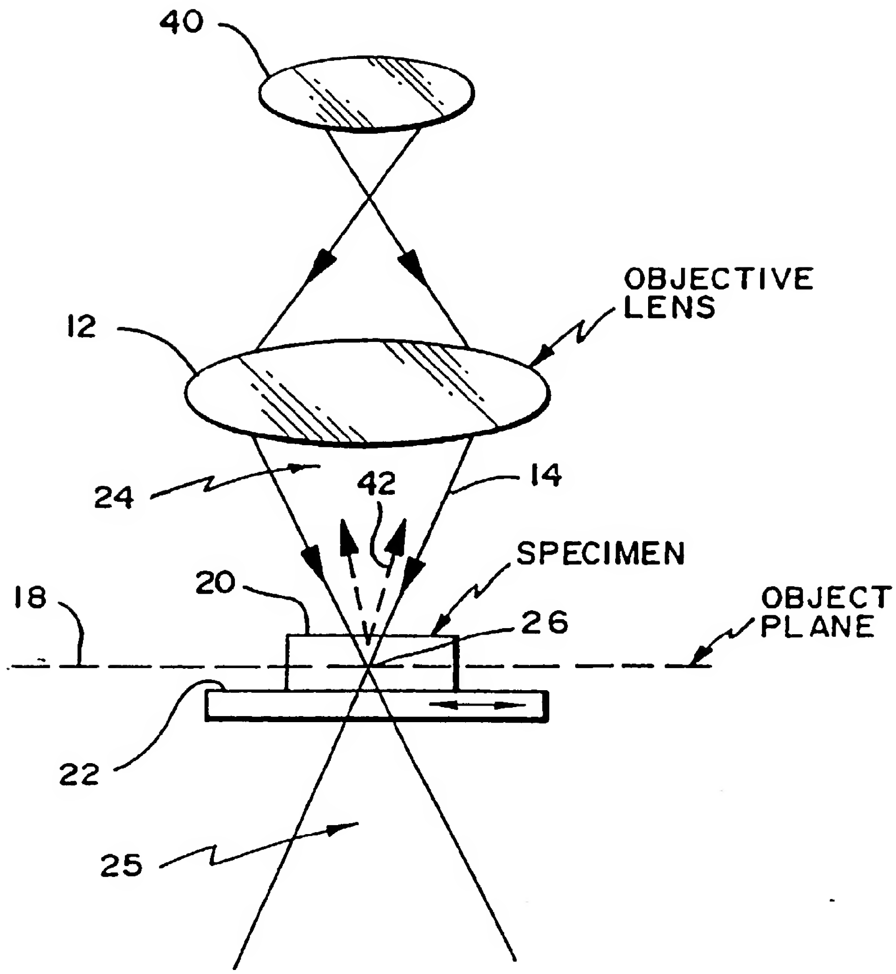

40
OBJECTIVE
LENS
12
24
42
14
SPECIMEN
20
18
OBJECT
PLANE
26
22
25

FIG. 1A

FIG.2

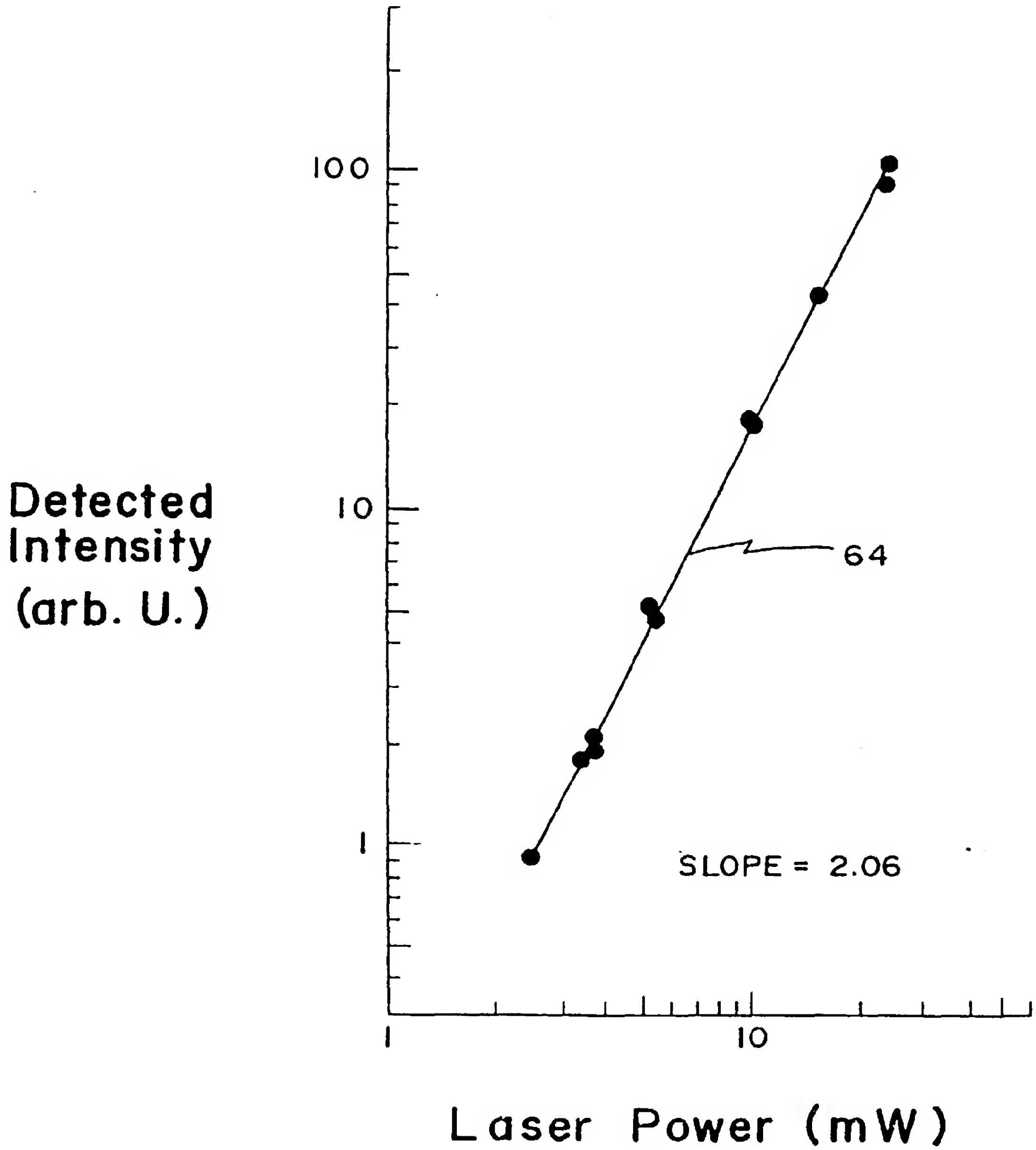

Detected
Intensity
(arb. U.)
100
10
1
1
10
64
SLOPE = 2.06
Laser Power (mW)

FIG. 3

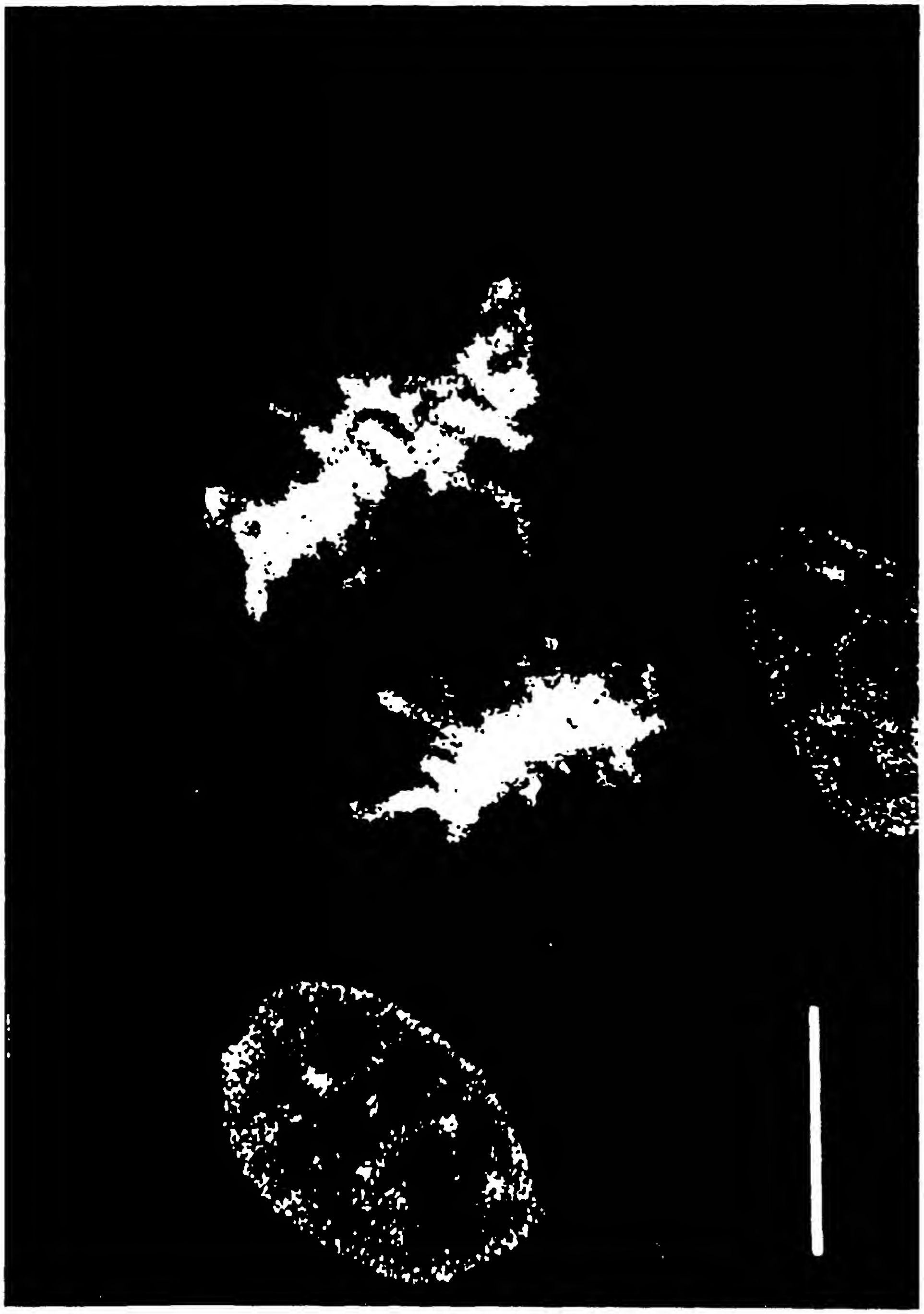


FIG.4

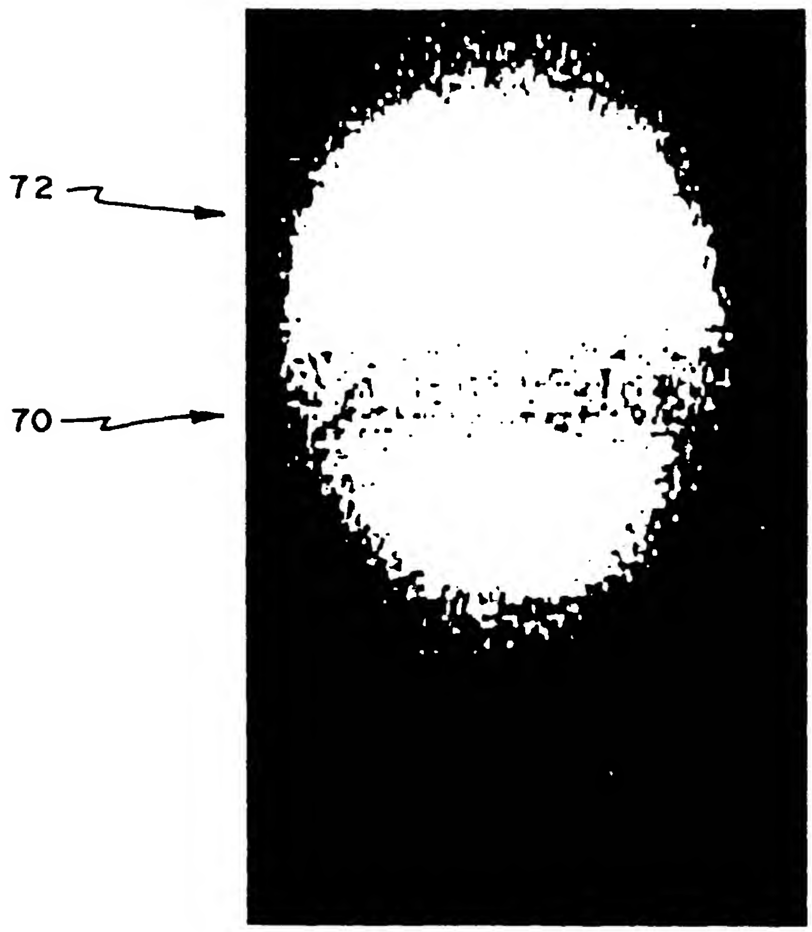


FIG.5

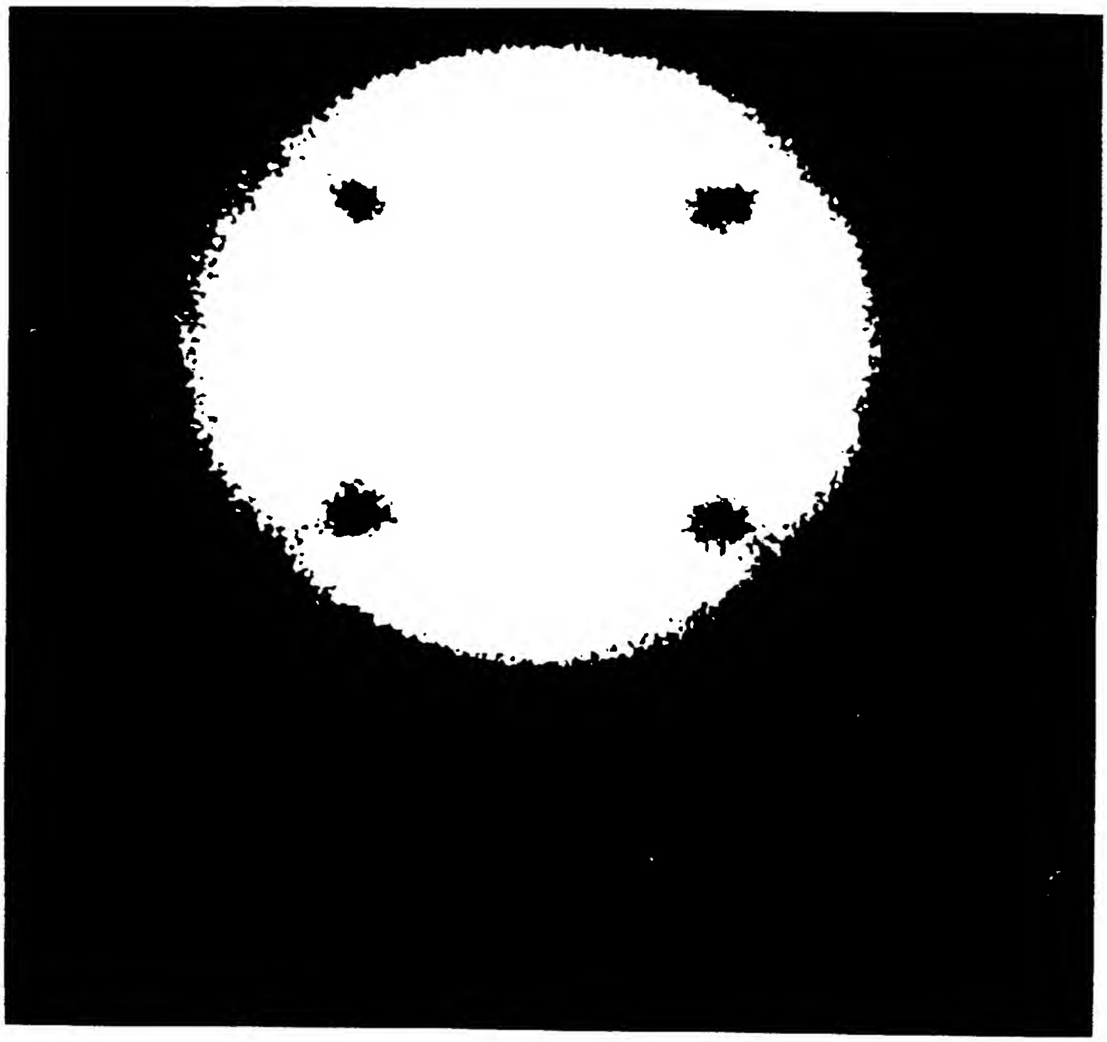


FIG. 6

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 97 10 8581

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
D,X	SCIENCE, 25 AUG. 1989, USA, vol. 245, no. 4920, ISSN 0036-8075, pages 843-845, XP002035947 PARTHENOPOULOS D A ET AL: "Three-dimensional optical storage memory" * the whole document *	1-3,7,8, 12	G02B21/00 G01N21/64 G11B7/00
Y	APPLIED OPTICS, 15 SEPT. 1978, USA, vol. 17, no. 18, ISSN 0003-6935, pages 2879-2882, XP002035948 SHEPPARD C J R ET AL: "Resonant scanning optical microscope" * page 2879, column 1 - column 2, paragraph 1; figures *	1-4,8,11	
Y	V.S.ANTONOV ET AL.: "Multiple Photon Processes in Molecules Induced by Picosecond UV Laser Pulses" 1983 , PICOSECOND PHENOMENA III, SPRINGER SERIES IN CHEMICAL PHYSICS , BERLIN, DE XP002035949 * page 310, paragraph 1 *	1-4,8,11	TECHNICAL FIELDS SEARCHED (Int.Cl.6)
A	US 4 288 861 A (SWAINSON WYN K ET AL) 8 September 1981 * column 1 - column 2 *	1,6-8,12	G02B G01N G11B
A	GB 1 236 108 A (WESTERN ELECTRIC COMPANY) 23 June 1971 * column 1 - column 2 *	1,6-8,12	
P,X	SCIENCE, vol. 248, no. 4951, 6 April 1990, pages 73-76, XP000381741 DENK W ET AL: "TWO-PHOTON LASER SCANNING FLUORESCENCE MICROSCOPY" * the whole document *	1-12	

-/--

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	24 July 1997	Scheu, M

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 97 10 8581

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
T	OPTIK, vol. 86, no. 3, 1 December 1990, pages 104-106, XP000307463 SHEPPARD C J R ET AL: "IMAGE FORMATION IN TWO-PHOTON FLUORESCENCE MICROSCOPY" * page 104 - page 106 * -----		
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	24 July 1997	Scheu, M

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P04C01)